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Development and Validation of a Stability-Indicating RP-HPLC Method for Related Substances in Dolutegravir Dispersible Tablets

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ABSTRACT

A rapid, simple, and robust reverse-phase HPLC method was developed and validated for the quantification of related substances in dolutegravir 10 mg dispersible tablets. This study primarily aimed to establish a new RP-HPLC method to quantify impurity B (a degradation impurity) as a related substance, following USP guidelines. Chromatographic separation was performed using a phenyl-hexyl column (250 × 4.6 mm, 5µ) with a mobile phase consisting of 45% buffer (sodium dihydrogen phosphate dihydrate and EDTA), 49% methanol, and 6% acetonitrile, adjusted to a pH of 2.5 ± 0.05 with orthophosphoric acid. The method used isocratic elution at a flow rate of 1.2 mL/min and maintained a column temperature of 35 °C, with detection at 258 nm using a PDA detector. The method was evaluated for stability, specificity, ruggedness, precision, linearity, accuracy, and robustness, according to USP standards, and showing high resolution and short retention times. All system suitability and validation parameters met the required criteria. The method showed excellent sensitivity, with LOD and LOQ values confirming its capability. Linearity was confirmed for both dolutegravir and impurity B with a correlation coefficient greater than 0.997. The recovery of the impurity was consistent and ranged from 80 to 120%. Overall, the method was found to be accurate and reliable for the determination of related substances in dolutegravir 10 mg dispersible tablets.

Keywords: RP-HPLC, Dolutegravir, Validation, Method development, Related substances

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Introduction

Dolutegravir is an antiretroviral medication classified as an integrase inhibitor, which targets and inhibits the HIV enzyme integrase. By blocking the function of this enzyme, dolutegravir effectively disrupts the replication process of HIV, potentially reducing viral load in the body [1-4]. The US Food and Drug Administration (FDA) has approved this drug, which can be used in combination with Rilpivirine (brand name Edurant) to treat HIV. The chemical structure of dolutegravir is presented in **Figure 1**, and its chemical name is the sodium salt of (4R,12aS)-N-(2,4-difluorobenzyl)-7-hydroxy-4-methyl-6,8-dioxo-3,4,6,8,12,12a-hexahydro-2H-pyrido[1’,2’:4,5]pyrazino[2,1-b][1,3]oxazine-9-carboxamide. It has a molecular formula of C20H19F2N3O5Na and a molecular weight of 419.38 g/mol.

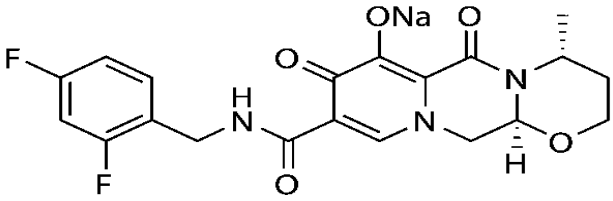


Figure 1. Structure of dolutegravir sodium

Dolutegravir (DTG), marketed as Tivicay, is a widely used antiretroviral drug prescribed for the treatment of HIV/AIDS, often combined with other medications. It also plays a role in post-exposure prophylaxis to reduce the risk of HIV transmission following potential exposure. In pharmaceutical manufacturing, monitoring impurities in drug substances and final products is a critical regulatory requirement. Even the smallest amounts of contaminants or solvents can impact the drug's therapeutic effectiveness and cause harmful side effects [5-7]. To accurately assess the impurity profile of dolutegravir, stability-indicating methods are necessary. High-performance liquid chromatography (HPLC) is an effective technique for separating, identifying, and quantifying individual components in a mixture, making it an essential tool in impurity detection. However, there is a lack of established methods in the literature for impurity analysis in dolutegravir products. This study presents a new RP-HPLC method specifically designed to quantify related substances in dolutegravir drug formulations. The method developed here is precise and reliable for determining the related impurities in dolutegravir dispersible tablets (10 mg).

Materials and Methods

Materials

The dolutegravir sodium standard and its related impurities were obtained from Jigs Chemicals, Ahmedabad. Methanol and acetonitrile, both of HPLC grade, were sourced from Rankem India Pvt. Limited. The study also employed dolutegravir dispersible tablets (10 mg) and ultrapure Milli-Q water.

Analytical method development

A new analytical approach for the detection of related substances in dolutegravir dispersible tablets (10 mg) was established following USP guidelines. A series of trials were conducted to identify the most suitable chromatographic parameters, such as varying buffer pH levels and adjusting the methanol and acetonitrile composition, using C-18, C-8, and Phenyl-Hexyl columns as stationary phases [8, 9]. The Phenyl-Hexyl column was specifically chosen due to its ability to provide distinct resolution between known and unknown degradation products. Compared to C-18 and C-8, this column demonstrated superior selectivity for aromatic compounds, attributed to its extended hexyl hydrocarbon functional group, which enhanced retention and separation. The optimized chromatographic conditions were achieved with a mobile phase comprising 45% buffer (sodium dihydrogen phosphate dihydrate and EDTA), 49% methanol, and 6% acetonitrile, adjusted to a pH of 2.5 ± 0.05 with ortho-phosphoric acid, under isocratic elution conditions. The flow rate was set at 1.2 mL/min, and the column temperature was maintained at 35 °C. The compounds were detected at 258 nm using a PDA detector. The chromatograms corresponding to the optimized method are displayed in **Figure 2**.

Optimized chromatographic conditions

Optimized Chromatographic Conditions are presented in **Table 1**.

Table 1. Optimized chromatographic conditions.

Column details	Kinetex® 5 µm Phenyl-Hexyl 100 Å, (250×4.6) mm. Make Phenomenex, Part No: 00G-4603-E0.
Flow rate	1.2 mL/min

Injection volume	20 µL
Column oven temperature	35 °C
Autosampler temperature	25 °C
Wavelength	258 nm
Run time	20 minutes
Wash vial	Water: Acetonitrile (1:1) % v/v, respectively.

Mobile phase/diluent preparations

Buffer preparation

1.56 g of Sodium dihydrogen orthophosphate dihydrate and 100 mg of EDTA were weighed and dissolved in 1000 mL of water, followed by thorough mixing. The solution was filtered through a 0.45 µm membrane filter. The pH was adjusted to 2.50 ± 0.05 using ortho-phosphoric acid, mixed well, and filtered again through a 0.45 µm nylon filter.

Mobile phase preparation

A mixture of the buffer solution, methanol, and acetonitrile was prepared in a ratio of 45:49:6 (v/v/v). The solution was sonicated for 5 minutes to remove any trapped air or gases.

0.1M hydrochloric acid solution

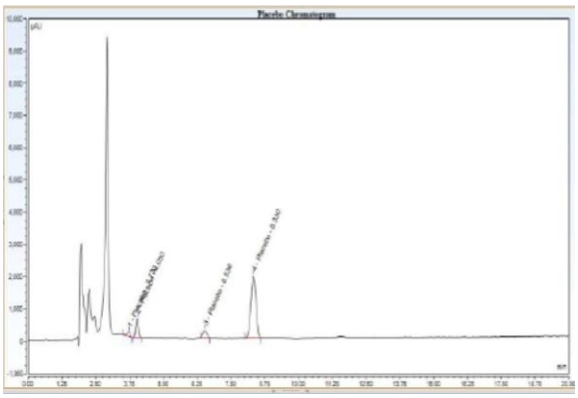
To prepare the hydrochloric acid solution, 8.5mL of concentrated hydrochloric acid was added to 500mL of water in a 1000mL volumetric flask. After mixing well, the solution was diluted to the final volume with water and mixed thoroughly.

Diluent preparation

A 1:1 (v/v) ratio of 0.1M hydrochloric acid and acetonitrile was mixed and sonicated for 5 minutes to degas the solution.

Blank

The diluent solution was used as the blank for this process.



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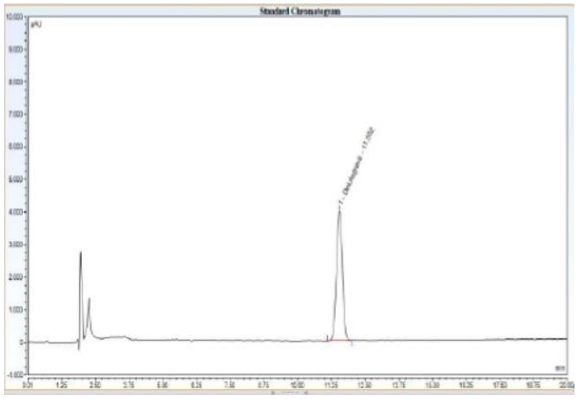

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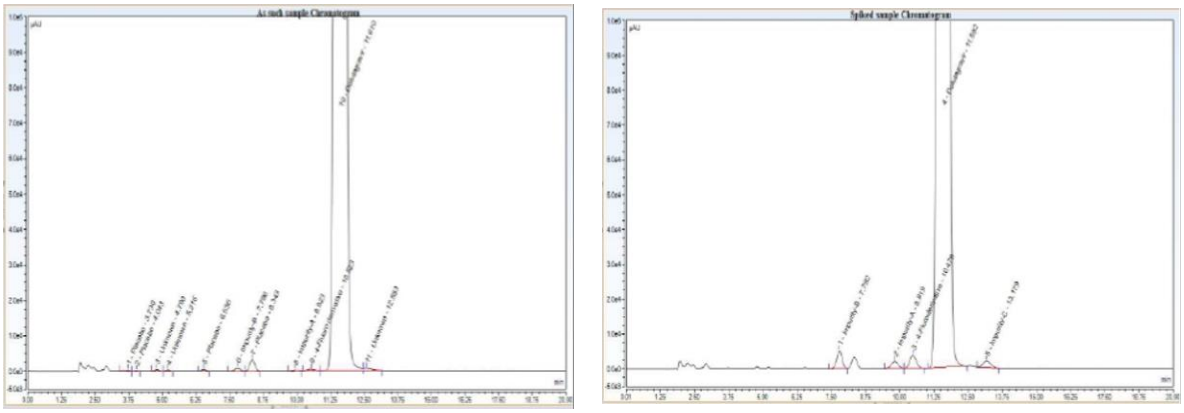


c) d)

Figure 2. Optimized chromatograms

Standard preparations

Standard stock 1 solution preparation

twenty mg of dolutegravir sodium standard, corresponding to 20 mg of dolutegravir, was weighed and added to a 100 mL volumetric flask. Then, 70 mL of diluent was introduced, and the mixture was sonicated for approximately 5 minutes until fully dissolved. The flask was filled to the 100 mL mark with diluent, ensuring proper mixing.

Standard stock 2 solution preparation

A 5.0 mL portion of the first standard stock solution was further diluted to 100 mL with diluent and mixed well.

Standard solution preparation (1.0 ppm)

5.0 mL of the second standard stock solution was transferred to a 50 mL volumetric flask and diluted to the mark with diluent. The solution was mixed thoroughly.

Impurity standard preparations

Impurity B stock solution

2.0 mg of impurity B was weighed and placed in a 20 mL volumetric flask, followed by the addition of 10 mL of diluent. The solution was sonicated until completely dissolved and then diluted to the final volume with diluent.

Impurity B standard solution

0.5 mL of the impurity B stock solution was diluted to 50 mL with diluent and mixed thoroughly.

Placebo preparation

Crushed placebo powder, equivalent to five tablets, was weighed and transferred into a 100 mL volumetric flask. 50 mL of diluent was added, and the flask was sonicated for 15 minutes. After cooling, the volume was adjusted to 100 mL with diluent, mixed well, and filtered through a 0.45 µm nylon filter, discarding the initial 5 mL.

Sample preparation (ten mg)

Crushed dolutegravir dispersible tablets (10 mg, equivalent to 5 tablets) were weighed and transferred to a 100 mL volumetric flask. 60 mL of diluent was added, and the mixture was sonicated for 15 minutes. After cooling, the solution was diluted to the mark with diluent and mixed well. The solution was filtered through a 0.45 µm nylon filter, discarding the first 5 mL.

Spiked sample preparation (10 mg)

Crushed dolutegravir dispersible tablets (10 mg, equivalent to five tablets) were weighed and added to a 100 mL volumetric flask. 70 mL of diluent was added along with 1.0 mL of impurity B individual stock. The solution was sonicated for 15 minutes and allowed to cool to room temperature. The flask was filled to the 100 mL mark with diluent and mixed well. The solution was filtered through a 0.45 µm nylon filter, discarding the first 5 mL.

Method validation procedure

The analytical method for related substances in dolutegravir dispersible tablets (10 mg) is validated by assessing the following parameters:

- Suitability of the system
- Specificity testing
  - Evaluation of blank, placebo, and impurity interference
  - Investigation of filter compatibility
  - Forced degradation study
- Precision assessment
  - Precision of the system
  - Method precision
  - Intermediate precision
- Stability of analytical solution and mobile phase
- Assessment of linearity
- Detection and quantification limits
- Evaluation of accuracy
- Determining the method's range
- Testing for robustness

System suitability

Before starting the analysis, it's vital to confirm the correct operation of the analytical system. The preparation of both blank and standard solutions followed the defined testing procedure.

Specificity

Specificity in an analytical method ensures the ability to accurately identify the target analyte even in the presence of other compounds such as impurities, by-products, or matrix components. The responses obtained from these solutions are summarized in **Table 2**.

Filter compatibility study

In this study, a placebo solution and a spiked sample solution were analyzed using filters made from nylon or PVDF with a 0.45 µm pore size. The first 3.0 mL and 5.0 mL of the filtrates from each solution were discarded. The Dolutegravir response and the percentage differences between the filters were recorded.

Forced degradation study

A forced degradation study was conducted to ensure that dolutegravir and its impurities remained unaffected by any degradation products that might emerge during stability testing or throughout shelf life. This study helped identify degradation mechanisms (oxidative, acid hydrolysis, or neutral hydrolysis). Blank and standard solutions were prepared as part of the procedure.

Precision

The precision of the analytical method is evaluated by comparing individual test results. This is typically reflected in the relative standard deviation (RSD) or coefficient of variation, which indicates how consistent the results are over multiple tests.

System precision

To assess the performance of the analytical system, a standard solution was injected, and six separate measurements were taken. Retention times and area responses were recorded, and the relative standard deviation was calculated for each. Gradient, blank, and standard solutions were injected to verify system accuracy.

Method precision

For method precision, a single batch of dolutegravir dispersible tablets (10 mg) was tested six times. This helps confirm whether the method produces consistent results across different batches. The percentages of known and unknown degradation products were calculated, along with the total degradation.

Intermediate precision

Intermediate precision examines how external factors, such as changes in equipment, analysts, or environmental conditions, affect the results. By adjusting one variable at a time, the method's robustness was reassessed, and results were compared to those from the original method precision test. Dolutegravir dispersible tablets were tested six times, and the percentages of specific and nonspecific degradation products were determined.

Linearity

Linearity measures the method's ability to produce results directly proportional to the concentration of the analyte within a defined range. Dolutegravir and impurity B were linearized from the limit of quantitation (LOQ) up to 200% of the target limit. The regression analysis of slope, intercept, and correlation coefficient was performed for the area response, with concentration plotted on the X-axis and area response on the Y-axis **(Table 3)**.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD is the lowest concentration of analyte that can be detected but not necessarily quantified, while the LOQ is the lowest concentration that can be quantified with precision and accuracy. These limits were determined using the signal-to-noise ratio method, and the area responses for both dolutegravir and impurity B were analyzed for their LOD and LOQ.

Accuracy

To evaluate the accuracy, the method was tested at three different levels in triplicate, including the LOQ concentration. Samples were prepared and injected at 50%, 100%, 150%, and 200% of the expected concentration, as well as at the LOQ. Recovery percentages for both dolutegravir and impurity B at these levels are presented in **Table 4**.

Range

The range of an analytical method is defined by the minimum and maximum concentration levels at which the method can accurately and linearly quantify the analyte. The analysis for dolutegravir's linearity and impurity B's precision was carried out, with calculations for the relative standard deviation (RSD), accuracy, and precision ranges for both substances.

Robustness

The robustness of a method assesses its reliability under small, controlled variations in testing conditions. This evaluation involved examining the effects of changes in parameters such as flow rate (± 10%), column temperature (± 5 °C), buffer pH (± 0.2 units), and the proportions of methanol and acetonitrile in the mobile phase (± 10%). The impact of these adjustments on the system's stability was recorded.

Results and Discussion

Method validation

System suitability

To assess the system's suitability, six injections of the standard solution were made, and the area responses were recorded. The following results were obtained: mean response of 62045.926, relative standard deviation (RSD) of 0.9%, theoretical plates of 18302, and a tailing factor of 1.04.

Data Interpretation: The data indicate that the system suitability criteria were satisfied, confirming that the system is operating within the required specifications.

Specificity

Table 2. Response of placebo, sample, and spiked sample solutions

Sample Name	Peak name	RT (Min)	RRT (about)	Peak purity
Blank	Blank	1.941, 2.208	NA	NA
Standard solution	Dolutegravir	13.420	NA	999
Placebo solution	Placebo	2.459,2.939,3.113,3.946, 4.219,7.439,9.613	NA	NA
Sample as such -10 mg	Impurity B	8.846	0.65	998
	Dolutegravir	13.513	1.00	1000
	Impurity D	12.046	0.89	992
	Unknown	4.426,5.306,10.953,12.486, 14.873	NA	NA
Spiked sample solution-10 mg	Impurity B	8.804	0.66	1000
	Dolutegravir	11.610		1000
	Impurity D	11.937	0.89	NA
	Impurity C	16.204	1.21	999
	Impurity A	11.937	NA	NA
	Unknown	4.424,5.277,5.817,10.884, 14.791	NA	NA

Data interpretation

From the results, it is evident that no interference occurred from the blank or placebo at the dolutegravir and impurity retention times. The purity of the key peaks satisfied the required criteria, confirming that the method is effective and specific for detecting related substances in dolutegravir dispersible tablets 10 mg.

Filter compatibility study

The area responses for the spiked and placebo solutions were recorded, and the percentage difference was determined for both unfiltered samples and the samples with 3 mL and 5 mL discarded filtrates, utilizing 0.45-micrometer Nylon and 0.45-micrometer PVDF filters.

Data interpretation

The results show that both the 0.45-micrometer Nylon and 0.45-micrometer PVDF filters are compatible for use in filtering the dolutegravir dispersible tablets 10 mg solution, with 5 mL of filtrate being discarded.

Forced degradation study

Table 3. % Assay, peak purity, and % mass balance for forced degradation

S. No.	Condition (sample)	Impurities from the RS method	% Assay	Peak purity	% Mass balance
1	Sample as such-I	0.154	98.3	1000	-
2	Alkali stressed sample_5N NaOH_4 Hours at 80°C	0.167	95.0	1000	96.7
3	Neutral stressed sample_Water_4 Hours at 80°C	0.470	98.9	1000	100.9
4	Thermal stressed sample_105°C_1 Hour	0.204	97.7	1000	99.4
5	Peroxide stressed sample_5%v/v_H_2O_2_ 4 hours at 80°C	0.275	98.0	1000	99.8
6	Sample as such-II	0.143	99.5	1000	-
7	Acid stressed sample_2N HCl_30 minutes at 80°C	10.485	89.8	1000	100.6
8	UV stressed Sample_16 hours at UV cabinet	0.154	97.3	1000	97.8
9	Photostability stressed sample (Visible) LUX stressed sample_1.2 million lux hours	0.701	97.5	1000	98.6

Analysis of Data: The degradation studies, performed under a variety of conditions (acidic, alkaline, peroxide, thermal, neutral, UV, and visible light), showed that dolutegravir was most vulnerable to acid-induced stress, as

indicated in **Table 3**. Despite this, the mass balance was consistently achieved across all conditions. This confirms the method's stability, making it suitable for assessing related substances in dolutegravir dispersible tablets (10 mg).

Precision

System precision

Six injections of the standard solution were carried out, and the response areas were measured. The retention time and peak area averaged 11.424 and 62035.926, respectively. The relative standard deviations (RSD) for these measurements were 0.2 for retention time and 0.9 for peak area.

Data Evaluation: Based on these results, the method demonstrates consistent performance in terms of retention time and peak area, as evidenced by the low RSD, meeting the system precision criteria.

Method and intermediate precision

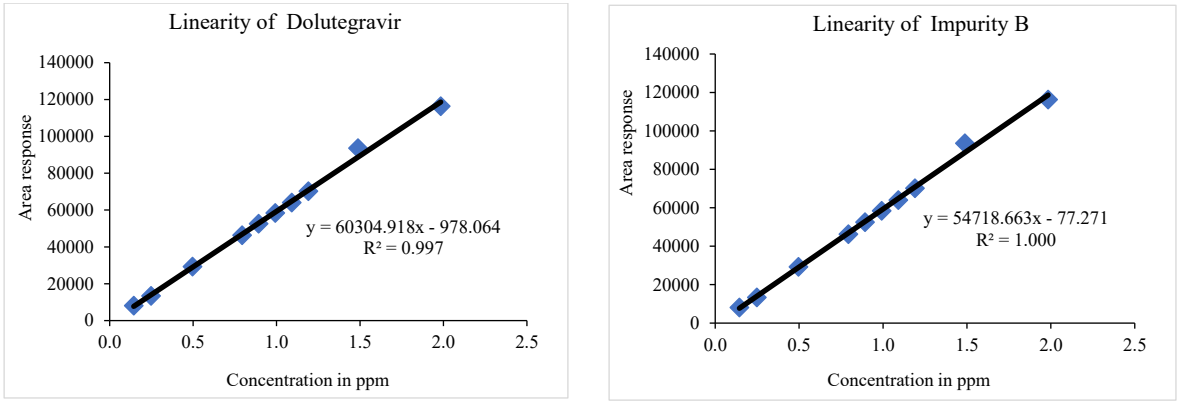
The total impurities, including impurity B and the largest unidentified impurity, were evaluated. The calculated mean impurity level was 0.29, with an RSD of 1.5.

Data Evaluation: These findings indicate that the method is stable and reproducible for determining related substances in dolutegravir dispersible tablets (ten mg).

Linearity

Table 4. Linearity of dolutegravir and impurity B

Linearity level in %	**Concentration in ppm**		**Peak area**	
	Dolutegravir	**Impurity B**	**Dolutegravir**	**Impurity B**
LOQ	0.0510	0.0523	3555.489	2806.664
10	0.1442	0.1544	8111.842	8337.053
25	0.2479	0.2558	13362.503	13703.251
50	0.4958	0.5115	29301.548	27605.915
80	0.7933	0.8185	46274.471	44277.477
90	0.8925	0.9208	52532.673	50506.496
100	0.9917	1.0231	58391.380	56894.493
120	1.1900	1.2277	70192.337	67637.253
150	1.4875	1.5346	93564.320	83786.201
200	1.9833	2.0462	116322.756	111442.089
	Dolutegravir		**Impurity B**	
Correlation coefficient ®	0.998		1.000	
Regression coefficient (r^2)	0.997		1.000	
Slope	60304.918		54718.663	
Intercept	-978.064		-77.271	
% Intercept	-1.7		-0.1	



a) b)

Figure 3. Linearity plot of dolutegravir and impurity B

Interpretation of Data: The relationship between the concentration of dolutegravir and impurity B and their corresponding detector responses is directly proportional across the range from the limit of quantification (LOQ) to 200% of the specified limit. Both the correlation and regression coefficients exceeded 0.995 and 0.990, respectively. The plotted data **(Figure 3)** of concentration (ppm) against area response under the curve further supports this linearity. Additionally, the percentage intercept at the 100% level remains within the allowable ± 5.0%. These results demonstrate that the detector's response is linearly related to the concentrations of both dolutegravir and impurity B.

Limit of detection (LOD) and limit of quantification (LOQ)

Interpretation of Data: Based on the linearity analysis, the LOD and LOQ were determined to be 2% (0.02 ppm) and 5% (0.05 ppm), respectively. This confirms that dolutegravir and impurity B are detectable and distinguishable at the LOD level. The signal-to-noise ratios for both the LOD and LOQ levels were consistent with the predefined acceptance criteria **(Table 5)**.

Accuracy

Table 5. Accuracy results

Sr. No.	Level	Area response	mg Added (actual)	mg recovered	% Recovery	Mean % recovery	% RSD
Accuracy for dolutegravir standard							
1	LOQ	3615.189	0.0052	0.0062	119.4	121.7	1.9
2		3330.135	0.0052	0.0057	121.9		
3		3585.911	0.0052	0.0061	123.9		
1	50%	28926.182	0.0518	0.0513	99.0	97.9	1.6
2		28819.656	0.0518	0.0511	98.6		
3		28091.431	0.0518	0.0498	96.1		
1	100%	57965.946	0.1035	0.1028	99.3	98.7	0.7
2		57678.986	0.1035	0.1023	98.8		
3		57150.173	0.1035	0.1014	98.0		
1	200%	116172.218	0.2070	0.2060	99.5	99.4	0.2
2		115749.264	0.2070	0.2053	99.2		
3		116275.024	0.2070	0.2062	99.6		
Accuracy for dolutegravir sample (spiked sample)							
1	LOQ	13458.061	0.0052	0.0040	92.0	92.0	1.4
2		13587.952	0.0052	0.0043	90.7		
3		13553.000	0.0052	0.0042	93.3		
1	50%	38966.179	0.0515	0.0506	98.3	98.1	0.2

2		38894.463	0.0515	0.0504	98.0		
3		38932.228	0.0515	0.0505	98.1		
1		67419.720	0.1029	0.1030	100.1		
2	100%	66871.834	0.1029	0.1020	99.1	99.4	0.6
3		66841.316	0.1029	0.1020	99.1		
1		122252.274	0.2058	0.2041	99.2		
2	200%	122600.746	0.2058	0.2048	99.5	99.3	0.2
3		122172.356	0.2058	0.2040	99.1		

Data Analysis: Based on the data presented, it is evident that the analytical method adheres to the predefined accuracy standards outlined in the protocol. Therefore, the method is confirmed as accurate for the analysis of related substances in dolutegravir dispersible tablets ten mg.

Range

The range was determined by assessing the RSD, linearity, and accuracy at various concentration levels, including LOQ, 50%, 100%, 150%, and 200%, for both dolutegravir and impurity B.

Data Analysis: The results indicate that the method provides consistent linearity and accuracy from LOQ up to 200% of the defined specification limit for dolutegravir and impurity B.

Robustness

Robustness testing was conducted by deliberately altering conditions such as the flow rate (± 10%), column temperature (± 5 °C), pH (± 0.2 units), methanol concentration (± 10%), and acetonitrile concentration (± 10%). The system suitability metrics, including % RSD, theoretical plates, USP tailing factor, and the relative retention time of impurity B, were evaluated.

Data Analysis: The method demonstrated robustness when subjected to controlled variations in flow rate, column temperature, pH, and the concentration of methanol and acetonitrile in the mobile phase, based on the analysis of system suitability parameters.

Conclusion

The RP-HPLC method for analyzing related substances in dolutegravir dispersible tablets 10 mg has been successfully developed and validated following USP guidelines and specifications. The method demonstrated characteristics of stability, specificity, robustness, precision, linearity, accuracy, and high resolution with a short retention time. Therefore, this method is suitable for its intended application.

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Conflict of Interest: None

Financial Support: None

Ethics Statement: None

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